Natural polysaccharides promote chondrocyte adhesion and proliferation on magnetic nanoparticle/PVA composite hydrogels

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ABSTRACT

This paper aims to investigate the synergistic effects of natural polysaccharides and inorganic nanoparticles on cell adhesion and growth on intrinsically cell non-adhesive polyvinyl alcohol (PVA) hydrogels. Previously, we have demonstrated that Fe_2O_3 and hydroxyapatite (nHAP) nanoparticles are effective in increasing osteoblast growth on PVA hydrogels. Herein, we blended hyaluronic acid (HA) and chondroitin sulfate (CS), two important components of cartilage extracellular matrix (ECM), with Fe_2O_3/nHAP/PVA hydrogels. The presence of these natural polyelectrolytes dramatically increased the pore size and the equilibrium swelling ratio (ESR) while maintaining excellent compressive strength of hydrogels. Chondrocytes were seeded and cultured on composite PVA hydrogels containing Fe_2O_3, nHAP and Fe_2O_3/nHAP hybrids and Fe_2O_3/nHAP with HA or CS. Confocal laser scanning microscopy (CLSM) and cell counting kit-8 (CCK-8) assay consistently confirmed that the addition of HA or CS promotes chondrocyte adhesion and growth on PVA and composite hydrogels. Particularly, the combination of HA and CS exhibited further promotion to cell adhesion and proliferation compared with any single polysaccharide. The results demonstrated that the magnetic composite nanoparticles and polysaccharides provided synergistic promotion to cell adhesion and growth. Such polysaccharide-augmented composite hydrogels may have potentials in biomedical applications.

1. Introduction

Hydrogels are biomimetic materials in terms of high water content, biocompatibility and biofunctionality and thus have been widely studied as promising candidates for tissue engineering [1]. Numerous natural [2] and synthetic hydrogels [3], or a combination of both [4], have been developed. Natural hydrogels usually possess excellent biocompatibility and low toxicity, but with poor strength and toughness [5]. Synthetic hydrogels are flexible in chemical structures, functionalities and mechanical strength and toughness, but could be limited by poor biocompatibility and biofunctionality [6]. In order to fabricate scaffolds for load-bearing soft tissues, taking cartilage as an example, adequate mechanical properties have been recognized as important as the biocompatibility and biofunctionality [7,8].

Numerous strategies have been developed to create composite hydrogels with bioceramics or by blending natural and synthetic hydrogels in order to combine complementary properties to achieve ideal support to cell adhesion, proliferation and differentiation.

Polyvinyl alcohol (PVA) has been widely investigated for biomedical applications [9–11] due to its merits including excellent biocompatibility, mechanical strength and toughness, low friction and high lubricity [12,13]. However, its intrinsically cell non-adhesive nature provides poor support to cell growth and integration to peripheral tissues [14,15]. Modification of PVA with biomolecules such as arginine–glycine–aspartic acid (RGD) peptide [16], hydroxyapatite [17] or natural polysaccharide (chondroitin sulfate) has shown improvements in cell adhesion and growth [18]. For example, hydroxyapatite (HAP) is the main inorganic composition in bone and has good osteoconductivity. The incorporation of HAP into PVA hydrogel enhanced the MC3T3-E1 cell density with well spreading morphology [19]. On the contrary, iron oxide nanoparticles had little influence on chondrocyte phenotype, viability and the production of major cartilage matrix constituents [20,21]. The hybrid of magnetic iron oxide nanoparticles and hydroxyapatite had been demonstrated favorable to cell adhesion

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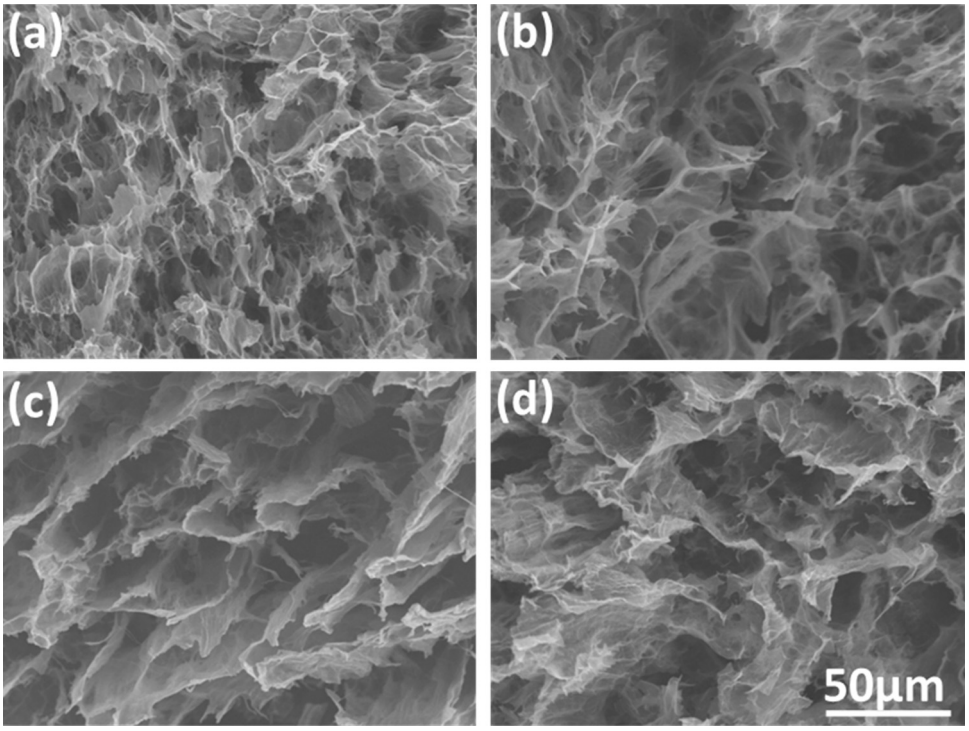

Fig. 1. SEM images of (a) PVA, (b) HA/PVA, (c) CS/PVA and (d) HA/CS/PVA.

and proliferation on composite hydrogels. For instance, magnetic scaffolds (FeHA/collagen) were fabricated by doping Fe^{2+}/Fe^{3+} ions into HAP nanocrystals nucleated on self-assembling collagen fibers. Such a composite hydrogel provided better support to cell adhesion, distribution and proliferation than the control HA/collagen scaffolds [22]. Our previous studies also demonstrated promoted osteoblast adhesion and proliferation on Fe_2O_3/HAP/PVA hydrogels than on HAP/PVA and PVA hydrogels [23].

In contrast, polysaccharides such as hyaluronic acid (HA) and chondroitin sulfate (CS) are two important biomolecules in most tissues including cartilage extracellular matrix (ECM). They are critical for cell adhesion, differentiation, migration and tissue integration [24,25]. Hydrogels based on HA and CS or their derivatives have been widely investigated as scaffolds to engineer or repair tissues including cartilage or osteochondral defects [26–28]. Interestingly, aldehyde-modified CS conjugated with amines could act as strong bio-adhesives to glue tissue interface in cartilage defect of rats [25]. Moreover, a combination of HA and CS has been demonstrated to achieve better results of cell growth than single HA or CS. For instance, compared with hyaluronic acid/silk fibroin (HA/SF, 20/80) (w/w) scaffolds, chondroitin sulfate/hyaluronic acid/silk fibroin (CS/HA/SF, 5/15/80) (w/w/w) scaffolds improved the angiogenesis and collagen production and promoted dermis regeneration [29]. Despite of these advantages of polysaccharides in cell growth, extracellular matrix secretion and tissue repair, inadequate mechanical properties remain as major limits to applications in tissue engineering [30]. It is desired to develop strong and tough hydrogels with excellent bioactivities and biofunctionalities for biomedical applications.

In this article, we combined polysaccharides (HA and CS) with previously reported strong and tough Fe_2O_3/nHAP/PVA nanocomposite hydrogels in order to further enhance the biofunctionality of these hydrogels. HA, CS and a combination of both were incorporated into PVA hydrogels and nanocomposites prior to freeze-thawing. The morphology, swelling and mechanical properties of these composite hydrogels were investigated. Neat PVA hydrogels and those composited with Fe_2O_3, nHAP and Fe_2O_3/nHAP hybrids were used for chondrocyte adhesion and proliferation study. Confocal laser scanning microscopy (CLSM) and cell counting kit-8 (CCK-8) assay results demonstrated that an appropriate content of polysaccharides (HA) could significantly improve the adhesion and proliferation of cells. When single or combined polysaccharides were incorporated into PVA hydrogels, the influence of chondrocyte adhesion and proliferation on PVA hydrogels was studied. Finally, the synergistic effect of polysaccharides and nanoparticles in promoting the adhesion and proliferation of chondrocytes is discussed.

2. Materials and methods

2.1. Materials

Poly(vinyl alcohol) (PVA, degree of polymerization: 1750 ± 50, analytical reagent, ≥99.0%) was purchased from Sinopharm Chemical Reagent Co., Ltd. Hyaluronic acid sodium (HA, *M*w = 380 kDa) was obtained from Shan Dong Freda Biopharm Co., Ltd. Chondroitin sulfate (CS) was obtained from Shaanxi Sciphar Natural Products Co., Ltd. $FeCl_2{\cdot}4H_2O$ (≥99.0%), $FeCl_3{\cdot}6H_2O$ (≥99.0%), $Ca(NO_3)_2{\cdot}4H_2O$ (≥99.0%), $(NH_4)_2HPO_4$ (≥99.0%) and NH_4OH (25.0%) solutions were of analytical reagent grade (Aladdin Chemistry Co., Ltd). All other chemicals and solvents were of analytical reagent grade.

2.2. Preparation of nanocomposite hydrogels and magnetic nanocomposite hydrogels

Magnetic Fe_2O_3/nHAP nanoparticles were prepared as described previously [23]. PVA was dissolved in deionized water with mechanical stirring at 90 °C for 6 h to yield a 3.3 wt% solution, which was then slowly cooled down to 25 °C. HA and CS were separately dissolved in deionized water with magnetic stirring at 25 °C for 2 h to yield a 2.0 wt% solution. Subsequently, single (HA or CS) or combined (HA and CS) polysaccharide solutions were, respectively, added into PVA solution to fabricate

polysaccharide/PVA (HA or CS was 5 wt% to PVA) solutions. The solutions were then added dropwise into cell culture plates, followed by six freeze-thawing cycles to produce polysaccharide/PVA hydrogels (marked as HA/PVA, CS/PVA and HA/CS/PVA). The Fe_2O_3/nHAP nanoparticle suspension was added into the polymer solutions (PVA, HA/PVA, CS/PVA and HA/CS/PVA), with mechanical stirring to generate homogeneous dispersions, which were then added dropwise into cell culture plates. The sols were freeze-thawed six times to produce magnetic nanocomposite hydrogels (coded as PVA-M, HA/PVA-M, CS/PVA-M and HA/CS/PVA-M). In order to further identify the contribution of the Fe_2O_3 and nHAP nanoparticles on chondrocyte adhesion and proliferation, Fe_2O_3/PVA and nHAP/PVA nanocomposite hydrogels with 50 wt% of nanoparticles were also fabricated.

2.3. Scanning electron microscopy (SEM)

The hydrogels were freeze-fractured in liquid nitrogen, and the fracture surface was sputtered with gold before imaging by using a Hitachi TM-1000 scanning electron microscope (SEM, Tokyo, Japan).

2.4. Transmission electron microscopy (TEM)

The PVA-M hydrogels were freeze-dried and cut into thin (~50 nm) slices at −60 °C. The slices were collected on copper grids coated with ultra-thin carbon film for transmission electron microscopy investigation by using an FEI Tecnai F20 instrument (TEM, Oregon, USA) at 200 kV.

2.5. Swelling behavior of hydrogels

The swelling behavior of hydrogels was assessed in deionized water at 25 °C. The freeze-dried hydrogels ($n = 5$) with 8 mm diameter and 4 mm height with a weight M_0 were used for the swelling experiments. The weight of swollen samples M_t was traced until equilibrium. The swelling ratio was defined as the mass ratio of the net liquid uptake to the dry hydrogel:

$$\text{Swelling ratio } (\%) = \frac{M_t - M_0}{M_0} \times 100\% \quad (1)$$

2.6. Porosity measurements of hydrogels

The porosity was measured by using a water replacement method. The freeze-dried hydrogel was put into a cylinder container with V_0 (mL) water, resulting in a total volume of V_1 (mL). Thus, the volume occupied by the xerogel was $(V_1 - V_0)$ mL. After equilibrium swelling, the hydrogels were taken out of the cylinder container, leaving V_2 (mL) water. Then, the pore volume of hydrogels was $(V_0 - V_2)$ mL, whereas the total volume of hydrogels was $(V_1 - V_2)$ mL. The porosity of hydrogels (P) could be calculated as

$$P(\%) = \left[\frac{(V_0 - V_2)}{(V_1 - V_2)}\right] \times 100\% \quad (2)$$

2.7. Compressive tests of hydrogels

Cylindrical hydrogel samples (approximately 8 mm in diameter and 4–6 mm height) were tested in unconfined compressive mode by using an Instron 5567 mechanical testing machine (Instron, MA, USA). Five samples were tested for each hydrogel ($n = 5$). The samples were tested at 10% strain/min. The compression limit was set as 98% strain to protect the load cell.

2.8. In vitro *cell culture with hydrogels*

Human chondrocytes (CHON-001, ATCC® CRL-2846™) were separately seeded on hydrogels to determine the cell adhesion and proliferation behaviors. Chondrocytes were grown in McCoy's 5A medium (Gibco), then supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin under a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. The culture medium was replaced twice a week. The cells were passaged by trypsinization. Cells at passage 3 were used for the experiments.

Hydrogels were cut into 8 mm diameter circular discs and then sterilized by immersing in 75% ethanol and followed by washing in sterile PBS. The cells (5×10^4 cells/mL) were seeded to the hydrogels ($n = 3$) in 48-well plates (Corning).

After culturing for specific days, the hydrogels were removed from the culture media and washed three times with PBS and then fixed with 2% glutaraldehyde for 12 h. The hydrogels were successively washed with PBS, soaked in 0.1% Triton X-100 for 10 min and washed again with PBS. Subsequently, the chondrocytes on the hydrogels were blocked with 1% bovine serum albumin (BSA) for 30 min, stained with 50 μg/mL phalloidin-FITC (Invitrogen, St Louis, USA) solution for 1 h at 25 °C and then washed with PBS several times to remove unbounded phalloidin conjugate. Then, 10 μg/mL 4′,6-diamidino-2-phenylindole (DAPI) (BestBio, Shanghai, China) was added and incubated in dark at 25 °C for 5 min. Then the samples were washed with PBS. Finally, the cytoskeleton and nucleus of chondrocytes were imaged by using a confocal laser scanning microscope (CLSM) (Leica TCS SP5 II, Braunschweig, Germany) or a fluorescence microscope (Olympus IX-51, Tokyo, Japan).

The chondrocytes on the hydrogels ($n = 5$) were quantitatively investigated by the CCK-8 assay after cultured for 1, 3, 7 and 15 days. After removal of the culture media from cell culture plates, 300 μL fresh culture media and 30 μL CCK-8 kit solutions were immediately added and homogeneously mixed and then incubated for 6 h in a CO_2 incubator. Finally, 200 μL reaction solutions were put into 96-well plate. The optical density of each well at 450 nm was read by a microplate reader (SpectraMax 190, Molecular Devices, USA).

3. Results and discussion

3.1. Morphology and structures of hydrogels

The hydrogels showed typical interconnected porous structures, which is beneficial for the mass and nutrient transport. The pore size of PVA hydrogel was 7.6 ± 2.2 μm (Fig. 1a). When the polysaccharides (5 wt% to PVA) were incorporated into PVA hydrogels, the pore size was increased gradually (Fig. 1b–d). The pore sizes of CS/PVA (5%/95%, w/w) and HA/PVA (5%/95%, w/w) hydrogels were 31.6 ± 4.0 and 10.3 ± 2.4 μm. In addition, the pore size of HA/CS/PVA (5%/5%/90%, w/w/w) was 16.6 ± 2.7 μm, which is between those for HA/PVA and CS/PVA hydrogels. With the presence of Fe_2O_3/nHAP nanoparticles (50 wt% to PVA), the pore size of PVA hydrogel was decreased to 5.2 ± 1.2 μm (Fig. 2a). The results were consistent with our former report [23]. Previously, for the Fe_2O_3/nHAP/PVA hydrogels, when the content of nanoparticles was 10, 20 and 50 wt%, the pore sizes were smaller than those of PVA hydrogels. During freeze-thawing, the porous structures were formed due to the phase separation between PVA and ice [13]. The hydroxyl groups on the surface of Fe_2O_3/nHAP nanoparticles and PVA may form hydrogen bonds. Thus, the nanoparticles may serve as nuclei to ice, leading to the formation of small ice crystals, which results in small pores in the composite PVA hydrogels [31]. TEM images demonstrated that the rod-like Fe_2O_3/nHAP nanoparticles were well distributed in the PVA hydrogels (Fig. 2e), which is critical for the mechanical properties of the hydrogels. When polysaccharides were added into PVA-M hydrogels, the pore size

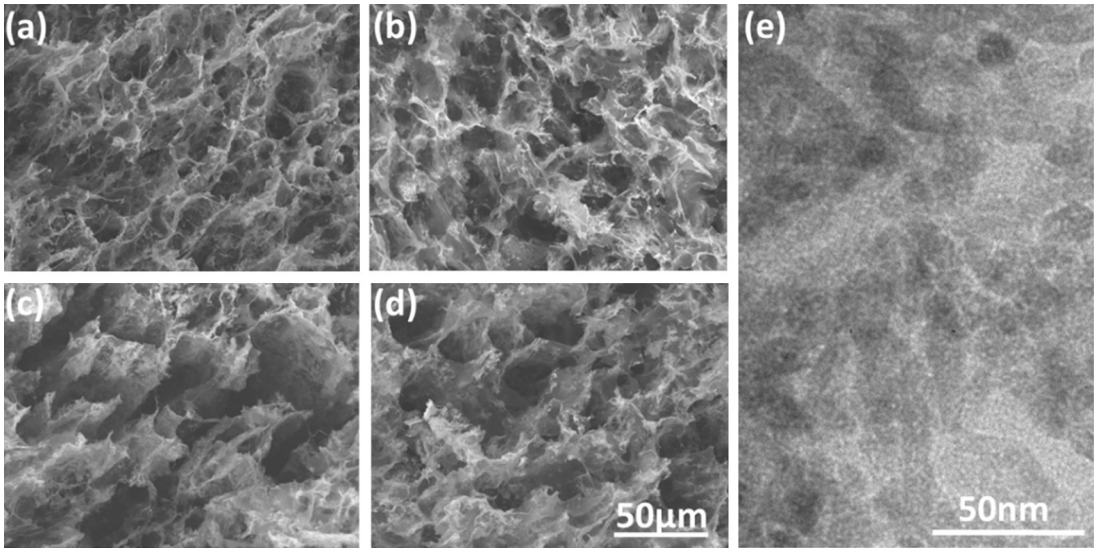

Fig. 2. SEM images of (a) PVA-M, (b) HA/PVA-M, (c) CS/PVA-M and (d) HA/CS/PVA-M and (e) TEM image of PVA-M.

was improved significantly (Fig. 2b–d). The pore sizes of HA/PVA-M, CS/PVA-M and HA/CS/PVA-M were 8.7 ± 2.0, 33.8 ± 6.9 and 15.9 ± 2.5 μm. The CS had the highest influence on pore sizes for PVA hydrogels, whereas the HA polysaccharides showed less influences. The pore size of HA/CS/PVA hydrogels was between that of HA/PVA and CS/PVA hydrogels. The porosity was measured at equilibrium swelling by using a water replacement method. The results (Supplementary Material, Fig. S1) showed that the incorporation of magnetic HAP nanoparticles into hydrogels reduced the porosity.

3.2. Swelling properties of hydrogels

The equilibrium swelling of PVA and PVA-M hydrogels was achieved by soaking in water for 24 h. The equilibrium swelling ratio (ESR) of PVA-M (50 wt% Fe_2O_3/nHAP nanoparticle with respect to PVA) ($305.3 \pm 10.4\%$) was decreased in comparison to neat PVA hydrogels ($365.7 \pm 22.4\%$).

When 5 wt% CS and/or HA was added into the PVA hydrogels, the swelling ratio was increased significantly (Fig. 3a). The swelling ratio of CS/PVA hydrogels was the highest ($1129.3 \pm 101.8\%$), while that of HA/PVA hydrogels was the lowest ($579.5 \pm 58.7\%$) and the ESR of HA/CS/PVA hydrogels was in between ($934.9 \pm 165.8\%$). Meanwhile, for PVA-M hydrogels, the addition of polysaccharides also increased the swelling ratio significantly (Fig. 3b). The swelling ratios of CS/PVA-M, HA/CS/PVA-M and HA/PVA-M hydrogels were 6.1, 2.3 and 1.3 times as those of PVA-M hydrogels. Moreover, CS induced higher water uptake in hydrogels than that of HA. The results may be attributed to the difference in osmotic pressures in these systems. The pH value of water solution was 7.0, which was higher than the pKa of CS (2.6) and HA (2.9) [32,33]. Thus CS and HA molecules in hydrogels are ionized, with the ionic strength of CS higher than that of HA. As a result, the osmotic pressure in PVA hydrogels with CS is higher than that with HA, which drives a higher water uptake or ESR for the former than for the latter [34].

3.3. Mechanical properties of hydrogels

Fig. 4 shows representative compressive engineering stress–strain curves and the compressive strengths of the hydrogels. The compressive strengths of PVA, HA/PVA (5%/95%, w/w), CS/PVA (5%/95%, w/w) and HA/CS/PVA (5%/5%/90%, w/w/w) hydrogels were 0.3 ± 0.1, 0.8 ± 0.3, 1.1 ± 0.4 and 2.7 ± 0.4 MPa. When nanoparticles were blended into PVA hydrogels, the compressive strength was increased significantly. For example, the compressive strength of PVA-M (50 wt% Fe_2O_3/nHAP nanoparticles) was 29.8 ± 7.3 MPa. For the nanoparticles/PVA composite hydrogels, the addition of polysaccharides increased the mechanical properties slightly. The compressive strengths of HA/PVA-M, CS/PVA-M and HA/CS/PVA-M were 25.8 ± 2.5, 28.6 ± 1.5 and 34.6 ± 7.1 MPa, respectively (Fig. 4b). These results showed that the nanoparticles played dominant roles in improving the mechanical properties of PVA hydrogels.

3.4. The influence of Fe_2O_3, nHAP and Fe_2O_3/nHAP nanoparticles on chondrocyte adhesion and proliferation on PVA hydrogels

In our previous study, Fe_2O_3, nHAP and Fe_2O_3/nHAP nanoparticles have been demonstrated to enhance osteoblast adhesion and growth on PVA hydrogels [23]. Herein, we further investigate the effect of these nanoparticles on chondrocyte adhesion and proliferation on PVA-based hydrogels. After culturing with chondrocytes for 2, 5 and 9 days, SEM and fluorescent microscopy were used to evaluate the cell morphology, adhesion and proliferation behaviors. As shown in Supplementary Material, Figs. S2 and S3, there were few chondrocytes on PVA hydrogels, and the chondrocytes did not proliferate over time. In contrast, chondrocytes nicely adhered and grew on Fe_2O_3/PVA hydrogels. Moreover, the chondrocyte density on the PVA-M hydrogel was higher than that on nHAP/PVA hydrogels and less than that on Fe_2O_3/PVA hydrogels. In the following study, Fe_2O_3/nHAP composite nanoparticles were used as model nanoparticles to composite with PVA hydrogels.

3.5. The influence of polysaccharides on chondrocyte adhesion and proliferation on PVA and PVA-M hydrogels

3.5.1. The influence of HA content on chondrocyte adhesion and proliferation on PVA hydrogels

Hyaluronic acid (HA) is a linear high molecular weight polysaccharide and the backbone of glycosaminoglycan (GAG) and has been demonstrated to regulate cell adhesion, proliferation and differentiation. Moreover, HA plays a very important role in lubricating the moving surface of knees [35]. Herein, HA was used to promote the cell adhesion and proliferation on PVA hydrogels.

PVA hydrogels containing 5%, 10% and 25% (w/w) HA, denoted as 5HA/PVA, 10HA/PVA and 25HA/PVA, were seeded with chondrocytes and cultured for 1, 3, 7 and 15 days. Fig. 5 shows the

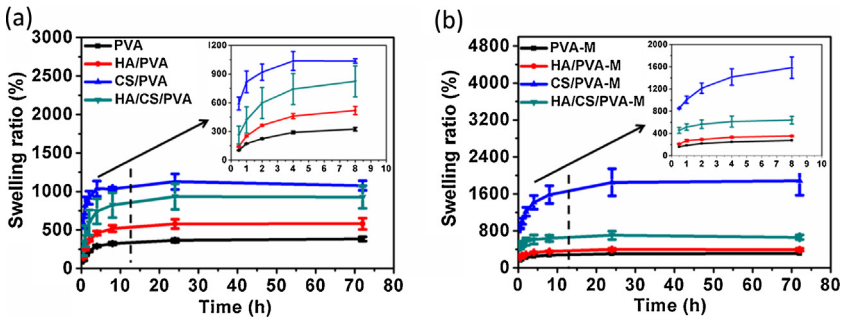

Fig. 3. Swelling properties of (a) polysaccharides/PVA composite hydrogels and (b) polysaccharides/nanoparticles/PVA composite hydrogels. (a) The zoom-in view of and (b) within the first 10 h. The error bars represent deviation with $n = 5$ for each sample.

CLSM images of the chondrocytes. There were few chondrocytes on PVA hydrogels at day 1 and no obvious chondrocyte proliferation from 3 to 15 days (Fig. 5a–d), which indicates that PVA hydrogels are not conducive to cell adhesion and proliferation. When 5% HA was added into PVA hydrogels, the chondrocyte densities on 5HA/PVA hydrogels were increased significantly in 7 and 15 days. However, the adhesion and proliferation of chondrocytes on 10HA/PVA and 25HA/PVA hydrogels were not different from those on PVA hydrogels.

In contrast, the cell proliferation for 1, 3, 7 and 15 days was also studied by using CCK-8 assay (Fig. 6). The results showed that the proliferation of chondrocytes was the best on 5HA/PVA hydrogels. When the HA content was increased to 10% and 25%, the cell adhesion and proliferation on HA/PVA hydrogels were no better than those on PVA hydrogels. The results of CCK-8 assay were consistent with the fluorescent observations (Fig. 6). Some studies demonstrated that the influence of HA on secreting cartilage extracellular matrix appeared dose-dependent. As the hyaluronic acid (HA) was added into poly (ethylene glycol) dimethacrylate (PEGDM) and collagen type I hydrogels, the low content HA (0.5%, w/w)-based hydrogels had better results in increasing ECM production than that of high content HA (1.0%, w/w)-based hydrogels [36]. Allemann et al. [37] prepared HA/collagen scaffolds with the content of HA of 2, 5, 10 and 14% w/w and seeded with chondrocytes. After 7 days culture, the chondrocytes in 2% HA/collagen scaffolds expressed more ECM than those in 10% HA/collagen scaffolds. Moreover, Akmal et al. [38] evaluated the effects of HA on chondrocytes. Compared with high concentrations of HA (2.0 and 3.0 mg/mL), the low concentrations of HA (0.1 and 1.0 mg/mL) considerably promoted the expression of DNA, sulfated glycosaminoglycan and hydroxyproline synthesis. In our study, the low content of HA (5%) was more beneficial for cell growth than the high content of HA (10% and 25%). Our results were consistent with those in the literature.

It is noted that, in contrast to the influence of HA content on chondrocyte adhesion and growth, the CS content (2, 11 and 25 wt%) showed no obvious promotion of chondrocyte adhesion and proliferation in comparison to PVA (Supplementary Material, Fig. S4). Therefore, for the following studies, the CS content was used as 5 wt% to match the optimal HA content of 5 wt%.

3.5.2. The impact of single and complex polysaccharides for chondrocyte adhesion and proliferation on PVA

Hyaluronic acid (HA) and chondroitin sulfate (CS) are the main components of cartilage ECM. The CS was connected to the backbone of HA. In order to create biomimetic environment, HA (5 wt%), CS (5 wt%) and HA (5 wt%)/CS (5 wt%) polysaccharides were introduced into PVA hydrogels, which were seeded and cultured

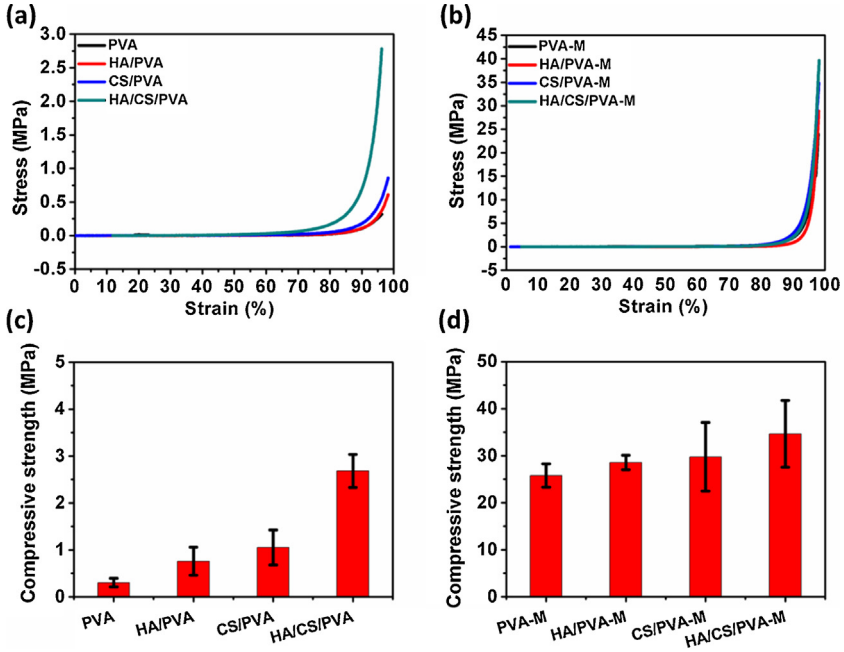

Fig. 4. Stress–strain curves of (a) polysaccharide/PVA and (b) polysaccharide/nanoparticle/PVA composite hydrogels. The compressive strength of (c) polysaccharide/PVA and (d) polysaccharide/nanoparticle/PVA composite hydrogels. The error bars represent standard deviation with $n = 5$ for each sample.

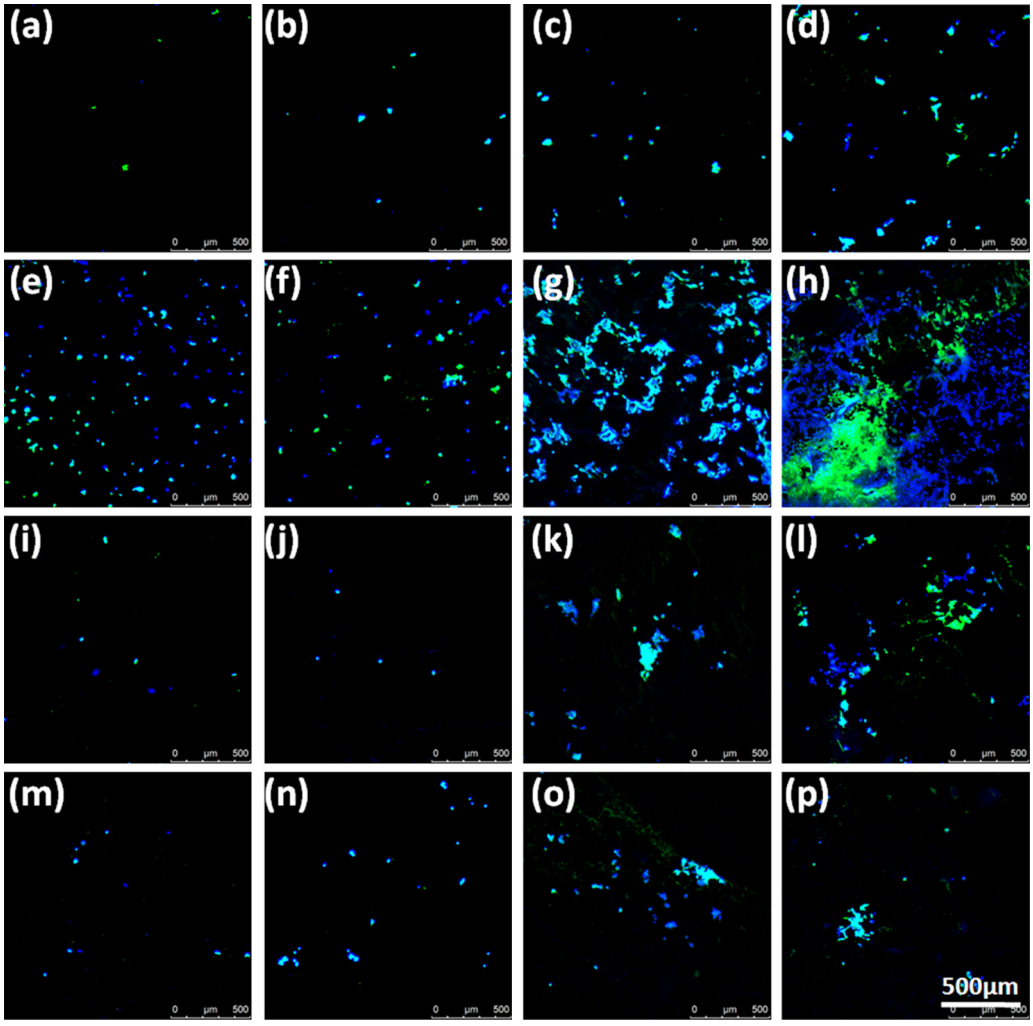

Fig. 5. Fluorescent photos of chondrocyte adhesion and proliferation on (a–d) PVA, (e–h) 5HA/PVA, (i–l) 10HA/PVA, (m–p) 25HA/PVA hydrogels; the cell culture times were (a, e, i, m) 1 day, (b, f, j, n) 3 days, (c, g, k, o) 7 days and (d, h, l, p) 15 days. Green: cytoskeleton stained by phalloidin-FITC; blue: nucleus stained by DAPI.

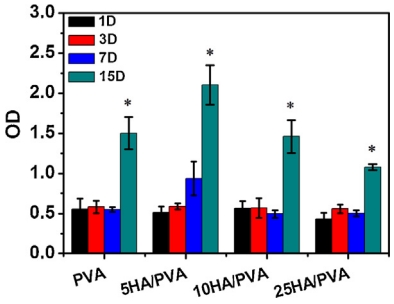

Fig. 6. Chondrocyte proliferation on PVA, 5HA/PVA, 10HA/PVA and 25HA/PVA hydrogels by CCK-8 assay. The error bars represent deviation with $n = 5$ for each sample. $^{*}P < 0.05$.

with chondrocytes. After culture for 3, 7 and 15 days (Fig. 7), the number densities of chondrocytes on the HA/PVA and CS/PVA hydrogels were higher than those on the PVA hydrogel. When HA and CS were co-mixed into PVA hydrogels, the chondrocyte adhesion and proliferation on HA/CS/PVA hydrogels were improved in comparison to those on HA/PVA or CS/PVA hydrogels (Fig. 7). These results showed that the co-existence of CS and HA showed better effects on chondrocyte growth than the single HA or CS biopolymers.

Similar synergistic effect of CS and HA was reported by Kaplan and co-workers [29] who fabricated silk fibroin (SF)/chondroitin sulfate (CS)/hyaluronic acid (HA) ternary scaffolds by freeze-drying. The SF, SF/HA and SF/CS/HA scaffolds were implanted onto dorsal full-thickness wounds of rats. Compared with SF and SF/HA scaffolds, the SF/CS/HA scaffolds accelerated dermis regeneration, angiogenesis and collagen deposition. In promoting dermal tissue regeneration, the polysaccharides (CS/HA) were better than single polysaccharide (HA). It is concluded that HA or CS polysaccharides could improve cell adhesion and proliferation ability on PVA hydrogels. Moreover, combined these two kinds of polysaccharides had superiority in increasing cell growth behaviors in contrast to single polysaccharides (HA or CS).

3.5.3. The synergistic effects of nanoparticles and polysaccharides in increasing chondrocyte growth

With the presence of nanoparticles, the chondrocyte adhesion and proliferation on PVA-M hydrogels were enhanced in comparison to PVA hydrogels (Fig. 8a and e). In contrast, the chondrocyte growth was also enhanced on HA/PVA, CS/PVA and HA/CS/PVA hydrogels (Fig. 8b–d). The nanoparticles and polysaccharides played key roles in increasing chondrocyte adhesion and proliferation. It would be interesting to further investigate the combined effects of nanoparticles and polysaccharides on promoting chondrocyte growth. When HA and Fe_2O_3/nHAP nanoparticles

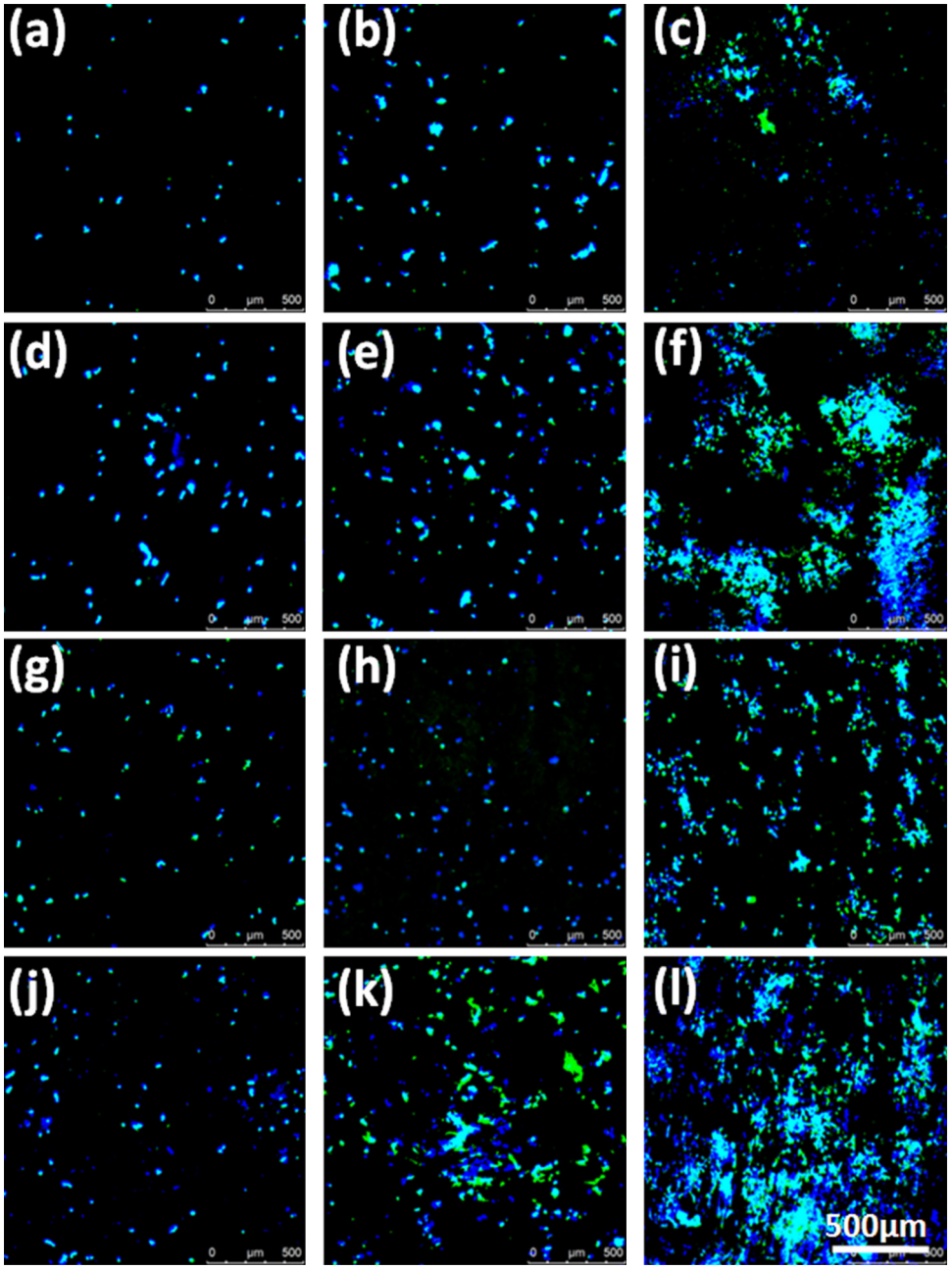

Fig. 7. Fluorescent photos of chondrocyte adhesion and proliferation on (a–c) PVA, (d–f) HA/PVA, (g–i) CS/PVA, (j–l) HA/CS/PVA hydrogels; the cell culture times were (a, d, g, j) 3 days, (b, e, h, k) 7 days and (c, f, i, l) 15 days. Green: cytoskeleton stained by phalloidin-FITC; blue: nucleus stained by DAPI.

were simultaneously added into PVA hydrogels, chondrocyte possessed preferential adhesion and proliferation ability on HA/PVA-M hydrogels in comparison to HA/PVA, PVA-M and PVA hydrogels. Besides, compared with CS/PVA, PVA-M and PVA hydrogels, the chondrocytes preferred to grow on CS/PVA-M hydrogels. Moreover, there was best chondrocyte adhesion on HA/CS/PVA-M hydrogels compared with HA/PVA-M and CS/PVA-M hydrogels. Thus, when the polysaccharides (HA, CS or HA/CS) and Fe_2O_3/nHAP nanoparticles were added into PVA hydrogels, the adhesion and proliferation of chondrocytes were improved considerably compared with polysaccharides/PVA, PVA-M and PVA hydrogels. These results demonstrate the synergistic effects of polysaccharides and nanoparticles in increasing chondrocyte adhesion and proliferation on PVA hydrogels.

The effect of scaffold pore size on chondrocyte growth and proliferation has been widely investigated on chitosan, chitosan–hyaluronic acid, gelatin and polycaprolactone scaffolds with pore diameters ranging from 10 to 500 μm. The results showed that chondrocyte growth was favorable on large pores (250–500 μm) in comparison to that on small pores (≤10 μm) [39–42]. In our case, the pore size of hydrogels ranged from 5 to 33 μm, which is smaller than that reported in the literature. In fact, in our experiments, there are no significant effects of pore size on chondrocyte adhesion and growth within this pore size range.

The routine compressive stress on the knee is about 3 MPa, whereas that on hip is 7–18 MPa [43]. Moreover, the compressive strength of cancellous bone is about 3–20 MPa [44]. The compressive strength values of our composite hydrogels are more than

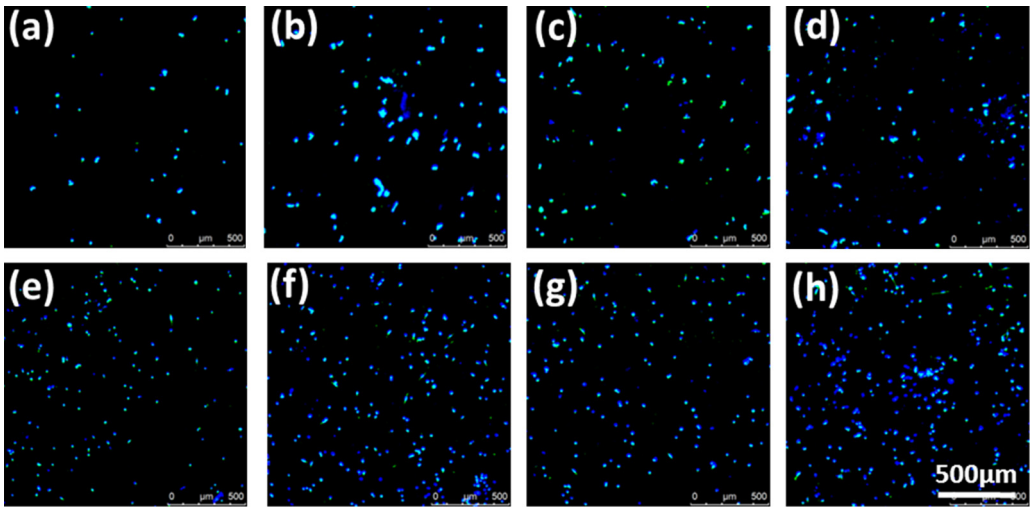

Fig. 8. CLSM images of chondrocytes on (a) PVA, (b) HA/PVA, (c) CS/PVA, (d) HA/CS/PVA, (e) PVA-M, (f) HA/PVA-M, (g) CS/PVA-M and (h) HA/CS/PVA-M hydrogels after 3-day culture. Green: cytoskeleton stained by phalloidin-FITC; blue: nucleus stained by DAPI.

25 MPa, which means that the hydrogels may sustain the routine loading when they were used as cartilage substitutes.

In this study, magnetic composite nanoparticles possessed the ability to improve cell adhesion and proliferation on PVA hydrogels. Hydroxyapatite nanoparticles could increase cell growth; this study was reported by Poursamar et al. [45], who adopted an *in situ* method to synthesize hydroxyapatite nanoparticles and fabricated hydroxyapatite and PVA composite scaffold by freeze-drying technique. The results showed that hydroxyapatite nanoparticles could support osteoblast growth. In this article, there were more chondrocyte adhesion and proliferation on magnetic nanoparticles and PVA composite hydrogels than on hydroxyapatite nanoparticles and PVA composite hydrogels. The Fe_2O_3 magnetic nanoparticles were incorporated into hydroxyapatite nanoparticles, improving the chondrocyte growth ability compared to hydroxyapatite nanoparticles. The similar trends were also confirmed by the research of Nhiem and Webster [46], who proved that Fe_3O_4 and hydroxyapatite nanoparticles presented better osteoblast growth behavior than hydroxyapatite nanoparticles alone.

Polysaccharides (HA and CS) are the main components of extracellular matrix (ECM) and have the ability to increase cell adhesion, proliferation and migration. In this study, single polysaccharides (HA or CS) added into PVA hydrogels could promote chondrocyte adhesion and proliferation on PVA hydrogels. In addition, combined polysaccharides (HA and CS) presented better chondrocyte growth behavior than single HA or CS polysaccharides in increasing chondrocyte growth. Furthermore, the synergistic effects between magnetic composite nanoparticles and polysaccharides could present better chondrocyte growth behavior than magnetic composite nanoparticles or polysaccharides.

4. Conclusions

Natural polysaccharides, *i.e.* HA and CS, have been introduced into freeze-thawed PVA and magnetic nanocomposite PVA hydrogels. The presence of polysaccharides dramatically increased the pore sizes and the equilibrium swelling ratio (ESR) of PVA hydrogels, without losing the mechanical strength. The adhesion and proliferation of chondrocytes on PVA-M hydrogels were significantly enhanced in comparison to Fe_2O_3/PVA and nHAP/PVA hydrogels. An optimal formulation of 5HA/PVA hydrogels has been demonstrated to best support chondrocyte adhesion and growth. Moreover, a combination of HA or CS into PVA effectively favors chondrocyte adhesion and proliferation, which appears superior to any single polysaccharide. Our results indicate synergistic effects of nanoparticles and polysaccharides in increasing chondrocyte adhesion and proliferation. The composite hydrogels of natural polysaccharides and magnetic PVA may have potentials in biomedical applications.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.colsurfb.2015.05.008

References

[1] N.A. Peppas, J.Z. Hilt, A. Khademhosseini, R. Langer, Adv. Mater. 18 (2006) 1345.
[2] S.J. Florczyk, K. Wang, S. Jana, D.L. Wood, S.K. Sytsma, J.G. Sham, F.M. Kievit, M. Zhang, Biomaterials 34 (2013) 10143.
[3] M.A. Daniele, A.A. Adams, J. Naciri, S.H. North, F.S. Ligler, Biomaterials 35 (2014) 1845.
[4] I. Strehin, Z. Nahas, K. Arora, T. Nguyen, J. Elisseeff, Biomaterials 31 (2010) 2788.
[5] W.C. Huang, K.H. Liu, T.C. Liu, D.M. Liu, S.Y. Chen, Acta Biomater. 10 (2014) 3546.
[6] M.P. Lutolf, J.A. Hubbell, Nat. Biotechnol. 23 (2005) 47.
[7] R.E. Wilusz, S. Zauscher, F. Guilak, Osteoarth. Res. Soc. 21 (2013) 1895.
[8] L.S. Wang, C. Du, W.S. Toh, A.C. Wan, S.J. Gao, M. Kurisawa, Biomaterials 35 (2014) 2207.
[9] P.M. Scholten, K.W. Ng, K. Joh, L.P. Serino, R.F. Warren, P.A. Torzilli, S.A. Maher, J. Biomed. Mater. Res. A 97A (2011) 8.
[10] D.A. Bichara, X. Zhao, H. Bodugoz-Senturk, F.P. Ballyns, E. Oral, M.A. Randolph, L.J. Bonassar, T.J. Gill, O.K. Muratoglu, Tissue Eng. A 17 (2011) 301.
[11] W.C. Hsieh, J.J. Liau, Carbohydr. Polym. 98 (2013) 574.
[12] J.A. Stammen, S. Williams, D.N. Ku, R.E. Guldberg, Biomaterials 22 (2001) 799.
[13] C.M. Hassan, N.A. Peppas, Macromolecules 33 (2000) 2472.
[14] S.A. Maher, S.B. Doty, P.A. Torzilli, S. Thornton, A.M. Lowman, J.D. Thomas, R. Warren, T.M. Wright, E. Myers, J. Biomed. Mater. Res. A 83A (2007) 145.
[15] M. Oka, Y.S. Chang, T. Nakamura, K. Ushio, J. Toguchida, H.O. Gu, J. Bone Joint Surg. 79 (1997) 1003.
[16] H. Kobayashi, Y. Ikada, Biomaterials 12 (1991) 747.

[17] T. Hayami, K. Matsumura, M. Kusunoki, H. Nishikawa, S. Hontsu, Mater. Lett. 61 (2007) 2667.
[18] S.J. Bryant, K.A. Davis-Arehart, N. Luo, R.K. Shoemaker, J.A. Arthur, K.S. Anseth, Macromolecules 37 (2004) 6726.
[19] W. Song, D.C. Markel, X. Jin, T. Shi, W. Ren, J. Biomed. Mater. Res. A 100 (2012) 3071.
[20] S. Ramaswamy, J.B. Greco, M.C. Uluer, Z.J. Zhang, Z.L. Zhang, K.W. Fishbein, R.G. Spencer, Tissue Eng. A 15 (2009) 3899.
[21] A.A. Appel, M.A. Anastasio, J.C. Larson, E.M. Brey, Biomaterials 34 (2013) 6615.
[22] A. Tampieri, M. Iafisco, M. Sandri, S. Panseri, C. Cunha, S. Sprio, E. Savini, M. Uhlarz, T. Herrmannsdorfer, ACS Appl. Mater. Interfaces 6 (2014) 15697.
[23] R. Hou, G. Zhang, G. Du, D. Zhan, Y. Cong, Y. Cheng, J. Fu, Colloids Surf. B 103 (2013) 318.
[24] D.J. Huey, J.C. Hu, K.A. Athanasiou, Science 338 (2012) 917.
[25] D.A. Wang, S. Varghese, B. Sharma, I. Strehin, S. Fermanian, J. Gorham, D.H. Fairbrother, B. Cascio, J.H. Elisseeff, Nat. Mater. 6 (2007) 385.
[26] A. Fakhari, C. Berkland, Acta Biomater. 9 (2013) 7081.
[27] T. Mikami, H. Kitagawa, BBA Gen. Subjects 1830 (2013) 4719.
[28] P. Chen, S. Zhu, Y. Wang, Q. Mu, Y. Wu, Q. Xia, X. Zhang, H. Sun, J. Tao, H. Hu, P. Lu, H. Ouyang, Biomaterials 35 (2014) 2827.
[29] S. Yan, Q. Zhang, J. Wang, Y. Liu, S. Lu, M. Li, D.L. Kaplan, Acta Biomater. 9 (2013) 6771.
[30] M. Garcia-Fuentes, A.J. Meinel, M. Hilbe, L. Meinel, H.P. Merkle, Biomaterials 30 (2009) 5068.
[31] A.K. Gaharwar, S.A. Dammu, J.M. Canter, C.-J. Wu, G. Schmidt, Biomacromolecules 12 (2011) 1641.
[32] A.R. Fajardo, L.C. Lopes, A.O. Caleare, E.A. Britta, C.V. Nakamura, A.F. Rubira, E.C. Muniz, Mater. Sci. Eng. C: Mater. 33 (2013) 588.
[33] J. Zhang, B. Senger, D. Vautier, C. Picart, P. Schaaf, J.C. Voegel, P. Lavalle, Biomaterials 26 (2005) 3353.
[34] J.A. Kelly, A.M. Shukaliak, C.C. Cheung, K.E. Shopsowitz, W.Y. Hamad, M.J. MacLachlan, Angew. Chem. Int. Ed. 52 (2013) 8912.
[35] A. Singh, M. Corvelli, S.A. Unterman, K.A. Wepasnick, P. Mcdonnell, J.H. Elisseeff, Nat. Mater. 13 (2014) 988.
[36] L.A. Callahan, A.M. Ganios, D.L. McBurney, M.F. Dilisio, S.D. Weiner, W.E. Horton, M.L. Becker, Biomacromolecules 13 (2012) 1625.
[37] F. Allemann, S. Mizuno, K. Eid, K. Yates, D. Zaleske, J. Glowacki, J. Biomed. Mater. Res. 55 (2001) 13.
[38] M. Akmal, A. Singh, A. Anand, A. Kesani, N. Aslam, A. Goodship, G. Bentley, J. Bone Joint Surg. 87B (2005) 1143.
[39] D.J. GriVon, M.R. Sedighi, D.V. SchaeVer, J.A. Eurell, A.L. Johnson, Acta Biomater. 2 (2006) 313.
[40] S. Yamane, N. Iwasaki, Y. Kasahara, K. Harada, T. Majima, K. Monde, S.I. Nishimura, A. Minami, J. Biomed. Mater. Res. 81A (2007) 586.
[41] S.H. Oha, I.K. Parka, J.M. Kimb, J.H. Lee, Biomaterials 28 (2007) 1664.
[42] S.M. Lien, L.Y. Ko, T.J. Huang, Acta Biomater. 5 (2009) 670.
[43] K.A. Athanasiou, E.M. Darling, J.C. Hu, Articular Cartilage Tissue Engineering, Morgan & Claypool Publishers Series, Zhejiang Province, PR China, 2010, pp. 8–9.
[44] A.F. Tancer, K.D. Johnson, Biomechanics in Orthopaedic Trauma: Bone Fracture and Fixation, Martin Dunitz, London, 1994, pp. 311.
[45] S.A. Poursamar, M. Azami, M. Mozafari, Colloids Surf. B 84 (2011) 310.
[46] T. Nhiem, T.J. Webster, Acta Biomater. 7 (2011) 1298.